Adsorption of Lysozyme onto the Silicon Oxide Surface Chemically Grafted with a Monolayer of Pentadecyl-1-ol

T. J. Su, R. J. Green, Y. Wang, E. F. Murphy, and J. R. Lu*

Department of Chemistry, University of Surrey, Guildford GU2 5XH, U.K.

R. Ivkov and S. K. Satija

NIST Centre for Neutron Research, Gaithersburg, Maryland 20899

Received December 2, 1999. In Final Form: February 11, 2000

The adsorption of chicken egg white lysozyme at the functionalized silicon oxide−solution interface has been studied using the combined measurement of spectroscopic ellipsometry and neutron reflection. The solid oxide surface was modified by coating a self-assembled monolayer of pentadecyltrichlorosilane with terminal hydroxyl groups (abbreviated to $C_{15}OH$). Neutron reflection measurement at the solid−D_2O interface showed that the $C_{15}OH$ layer was 16 ± 2 Å thick and the volume fraction was 0.94 ± 0.05, suggesting the formation of a close-packed monolayer. The adsorption of lysozyme was made at pH 4 and 7 with lysozyme concentration ranging from 0.03 to 4 g dm^{-3}. The results were then compared with those from previous studies at the hydrophilic SiO_2−water and the hydrophobed SiO_2−water interfaces, with the latter formed by coating a monolayer of octadecyl trichlorosilane (abbreviated to OTS). At 0.03 g dm^{-3} and pH 7 the surface excess was found to be 0.6 ± 0.3 mg m^{-2} at the $C_{15}OH$−water interface, as compared with 1.7 mg m^{-2} at the SiO_2−water interface and 1.9 mg m^{-2} at the OTS−water interface. As lysozyme concentration is increased to 4 g dm^{-3}, the surface excess at the $C_{15}OH$−water interface reaches 2.1 mg m^{-2}, as compared with 4.7 mg m^{-2} at the hydrophilic SiO_2−water interface and 5.1 mg m^{-2} at the OTS−water interface. These values demonstrate the attainment of the minimum surface excess on the hydroxyl surface. Shifting solution pH from 7 to 4 reduces adsorption on all the surfaces studied, but the lowest level of adsorption is again obtained on the hydroxyl surface. The reversibility of the adsorption at the $C_{15}OH$−water interface was examined by cycling the solution pH at different lysozyme concentrations. Adsorption was found to be completely reversible at the low lysozyme concentration of 1 g dm^{-3}, while at the high concentration of 4 g dm^{-3} the adsorption was irreversible.

Introduction

In a systematic investigation of the effect of surface hydrophobicity on the adsorption of proteins at the solid−solution interface, we have examined the adsorption behavior of a number of model proteins on the surfaces of hydrophilic and hydrophobed silicon oxide, with the latter being obtained by coating a self-assembled monolayer of octadecyl trichlorosilane (OTS).[1−5] As the contact angles for these two surfaces are about 0 and 110°, respectively, the results represent the studies under two extreme surface hydrophobicities. We found that while the OTS surface deteriorates the globular assembly of proteins, like egg white lysozyme, the globular framework is retained when these protein molecules are adsorbed on the hydrophilic silicon oxide. We have since extended this work to the examination of the effect of the surfaces with intermediate hydrophobicity. In this work we report the adsorption of lysozyme onto the surface of silicon oxide chemically grafted with a self-assembled monolayer of pentadecyltrichlorosilane with terminal hydroxyl groups ($Cl_3Si(CH_2)_{15}OH$, abbreviated to $C_{15}OH$). The advancing contact angle (θ_a) for the hydroxyl surface used in this work was 53 ± 3°.

Study of protein adsorption onto the surface containing terminal hydroxyl groups has a direct relevance to the understanding of the role played by these groups in the hydrophilic polymers. It has been known for a long time that surfaces bearing terminal hydroxyl groups can deter protein deposition.[6−9] Polymers comprising a large amount of hydroxyl groups are distinguished from other polymers by their capacity to uptake a high volume fraction of water. Polymers containing more than 30% water are usually referred to as hydrogels. A typical example of these hydrogel polymers is poly(2-hydroxyethyl methacrylate), which is widely known as HEMA polymer. The water content inside HEMA polymer can vary from 30 to 80 wt %, depending on its exact chemical nature and physical structure. Many natural polymers, e.g., globular proteins, are also strongly hydrated. Partly for this reason, synthetic hydrogels have been used as biomaterials capable of reducing unfavorable interactions between foreign materials and blood or tissues. Of various interactions, protein adsorption onto biomaterials is one of the primary events, leading to thrombus formation at the blood−biomaterial interface. The large volume fraction of water has been considered to be one of the main factors contributing to the reduction of protein adsorption on the surface of hydrogels.

* To whom correspondence should be addressed.

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10.1021/la991559j CCC: $19.00
Published on Web 04/21/2000

Many studies have been made to examine the behavior of hydrogels in blood. Although these studies represent the most relevant situations to the practical applications, the complexity of the composition of blood and the related physical and biological processes have made it difficult to evaluate the specific interaction between blood protein and the surface of the hydrogel. In addition, a hydrogel is usually a copolymer containing more than two components that differ in surface activity. As a result of possible preferential exposure to the outer surface, the exact surface composition of the hydrogel may respond to the change in solution environment and may therefore be different from its bulk value. Because of these complications, these types of studies can at most show that surfaces in contact with blood tend to become coated with protein in a very short time. Such work is certainly unable to reveal the adsorption behavior of a particular protein from blood or plasma. Although investigations have also been made to examine the adsorption of model proteins onto different solid substrates, most of these experiments have so far utilized polymeric particulate dispersions involving techniques such as light scattering, protein assays[10] and are therefore insensitive to the adsorbed protein layers. Techniques such as infrared, circular dichroism (CD) are capable of revealing information about the contents of α-helices and β-sheets, but they cannot quantify the amount of protein adsorbed.[11,12]

Neutron reflection has been shown to be powerful at revealing the density distribution profile of a protein layer along the surface normal direction. This information together with the dimension of globular proteins enables us to infer the *in situ* conformation of the adsorbed protein molecules. In our previous studies, we have used this technique to study the adsorption of a number of globular proteins at the hydrophilic silicon oxide−water interface[1−4] and found that adsorption of globular proteins such as lysozyme and albumins does not lead to the breakdown of their globular framework. The amount of protein adsorbed (surface excess) and the conformational structure of the adsorbed protein molecules show a strong correlation with solution pH and total ionic strength, suggesting that electrostatic interaction is a dominant force in governing the adsorption pattern. In contrast, when adsorbed at the hydrophobed solid−water interface lysozyme loses its globular framework and the adsorbed layer is represented by a fragmented density distribution, characteristic of the density profiles for synthetic polymers.[3]

In order to examine the effect of surface hydrophilicity in a much broader range, we have recently functionalized the surface of silicon oxide by chemically grafting a monolayer of pentadecyl-1-ol. This model surface allows a direct characterization of the effect of terminal hydroxyl groups on the structure and composition of the adsorbed protein molecules. A particular advantage of utilizing the self-assembled monolayer, instead of the polymeric surface formed by any copolymer, is that a clear correlation can be established between the surface composition and the adsorption behavior. Furthermore, since neutron reflection is particularly sensitive to the interfacial composition, the use of a relatively simple $C_{15}OH$ layer will reduce the level of complication in data analysis, hence enhancing the reliability of data interpretation. Both spectroscopic ellipsometry and neutron reflection have been used in this study. While spectroscopic ellipsometry is quick at following the time-dependent process, neutron reflection is more sensitive to the dimension of the adsorbed layers.

Experimental Section

Ellipsometric measurements were made on the Woollam Variable Angle Spectroscopic Ellipsometer 32 (WVASE32, manufactured by JA Woollam Co., Inc.) over a typical wavelength range of 350−650 nm. For silicon substrate, the most sensitive variation in ellipsometric signal occurs at the incidence angle around 75°, and for this reason we will show the data collected at 75° only. The measurements have been made at the solid−liquid interface using a specially constructed sample cell. The experimental geometry is such that the incoming beam enters the aqueous solution through a glass window and is reflected from the solid−water interface and exited from the opposite end of the glass window. The two windows on each side of the cell have been aligned at 75° with respect to the normal direction so that the incoming and exiting beams are always perpendicular to the window glasses. Thin glass slides were used as windows, and their effects on the incoming and exiting beams were calibrated at the beginning of the measurements.

Neutron reflection measurements were made using the NG-7 neutron reflectometer at the National Institute of Standards and Technology (NIST), Gaithersburg, MD,[13] using neutrons of wavelength of 4.75 Å. The range of momentum transfer, κ ($\kappa = (4\pi \sin \theta)/\lambda$), where λ is the wavelength and θ is the glancing angle of incidence) was provided by the variation of θ. The incoming beam was directed down toward the sample and was reflected from the interface upward and into the detector. A silicon block of dimensions $12.5 \times 5 \times 2.5$ cm^3 was used, and the solution was held in a Teflon cell that was clamped against the large polished face of the silicon block. The dimension of the beam at a given incidence angle was defined by sets of vertical and horizontal cadmium slits. The vertical slits set the width of the beam, and for the block used in this work the beam width was set at 28 mm. At a given incidence angle, the height of the beam was always maximized to optimize the beam intensity, but caution was taken not to overilluminate the sample surface.

Both ellipsometry and neutron reflection used the same coated block. The large (111) face of the silicon block was polished and cleaned using the same procedure as described previously.[1] The uniformity of the oxide surfaces was usually checked by ellipsometry over spots at different locations, and for the surface used in this work the oxide layer was always found to be 20 ± 5 Å thick if the refractive indices were taken to be the same as those corresponding to bulk SiO_2.

The freshly polished surface was then immersed in a solution of pentadecynyltrichlorosilane for 2 h using a mixed solvent of hexadecane and dichloromethane of the molar ratio of 1:1. The concentration of the coating reagent was fixed at about 1 mM, and the temperature was set at 11 ± 2 °C. The coated surface was then removed and rinsed with CH_2Cl_2, ethanol, and water before the whole block is dried in a dry nitrogen stream. The terminal double bonds were converted into hydroxyl groups by immersing the coated surface in BF_3 solution (Aldrich) for 5 min, followed by the exposure of the surface to alkaline hydrogen peroxide (29 wt %, Aldrich) for 30 min. The surface was subsequently rinsed with CH_2Cl_2, ethanol, and water. The procedure for the treatment is similar to that utilized by Sagiv et al.[14]

Chicken egg white lysozyme (Sigma, 95+%) was used as supplied. Lysozyme has an isoelectric point around pH 11. Its globular structure is roughly ellipsoidal and has an approximate dimension of $30 \times 30 \times 45$ Å^3. The solution pH was adjusted using phosphate buffer (Na_2HPO_4 and NaH_2PO_4), and the total ionic strength at each pH was fixed at 0.02 M. The small difference in the pH between H_2O and D_2O solutions was adjusted to be the same within an accuracy of 0.2 pH units. High-purity Elgastat water (UHQ) was used throughout the work, and D_2O was purchased from Sigma and was used as supplied. The surface tension of H_2O and D_2O was typically over 71 mN m^{-1} at 298 K, indicating the absence of any surface active impurity. The

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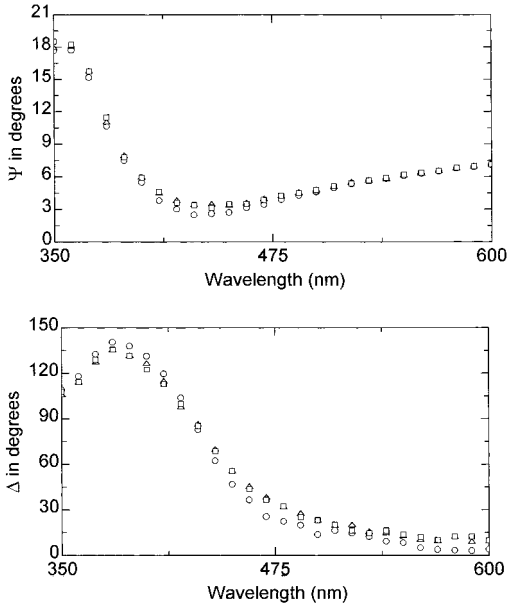

Figure 1. Ellipsometric profiles of Ψ (a) and Δ (b) recorded after 2 min (⊡) and 30 min (△) for the adsorption of 1 g dm^{-3} lysozyme at pH 7. The measurements at the $C_{15}OH$−buffered water interface (○) are also shown for comparison.

glassware and Teflon troughs for the measurements were cleaned using alkaline detergent (Decon 90) followed by repeated washing in UHQ water. Ellipsometry and neutron experiments were done between 22 and 26 °C.

Results

(A) Dynamic Adsorption Measured by Spectroscopic Ellipsometry. Protein adsorption is known to be time dependent. It may take several hours before the adsorption reaches a stable state. The time-dependent process is characterized by the transportation of protein molecules into the surface region and the subsequent process of structural reorientation within the adsorbed layer. Although a true equilibrium for protein adsorption may not be reached over a period of hours, the surface excess may reach a constant value in much shorter time scale. Ellipsometry is an appropriate technique for studying protein adsorption at the silicon oxide−solution interface, because the silicon substrate serves to highlight the adsorbed protein layer. A further advantage is that the measurement is fast and it only takes a few seconds to perform a single scan at a fixed angle and wavelength, thus allowing a close monitoring of the dynamic process of the adsorption. In a spectroscopic scan, however, it takes longer to acquire data points, but such measurement can improve the reliability of data interpretation. We show in Figure 1 the ellipsometric profiles recorded for the adsorption of 1 g dm^{-3} lysozyme at pH 7 onto the surface of silicon oxide chemically coated with a monolayer of $C_{15}OH$. In a spectroscopic measurement, the two ellipsometric angles, Ψ and Δ are usually recorded as a function of wavelength, λ. The angle Ψ measures the change in the amplitude of the polarized beam after reflection while Δ measures the change in phases.[15] The first set of Ψ and Δ was measured when the surface was in contact with the buffer solution (marked as circles). The second set of Ψ and Δ was recorded 2 min after the hydroxyl surface was in contact with the lysozyme solution (squares), and the third set, after 30 min (triangles). Clear difference can be seen between the first two measurements, but little difference is observed between the second and third sets of the data, suggesting that the adsorbed amount varies little after just 2 min.

Table 1. Lysozyme Surface Excesses at the $C_{15}OH$−Water Interface Measured by Spectroscopic Ellipsometry

pH	C/g dm^{-3}	Γ ± 0.4/mg m^{-2}	pH	C/g dm^{-3}	Γ ± 0.4/mg m^{-2}
4	0.03	0.4	7	0.03	0.7
	1	0.9		1	1.5
	4	1.6		4	2.1

Quantitative information about the variation of surface excess with time can be obtained by fitting refractive index profiles to Ψ and Δ simultaneously using the optical matrix formula. The variation of refractive index with λ is taken into account using the Cauchy equation.[15] Before lysozyme is adsorbed, there are already two layers on the surface of the solid: the native silicon oxide and the coated monolayer of $C_{15}OH$. The structure of the oxide layer was characterized at the solid−water interface before the organic layer was grafted. The thickness was found to be 20 ± 5 Å if the refractive index for the oxide layer was taken to be the same as that for the bulk silicon oxide. If it is assumed that coating of $C_{15}OH$ does not affect the thickness and composition of the oxide layer, the structure of the organic layer can be obtained by adding an extra layer in the model. The thickness of the $C_{15}OH$ layer was calculated to be 17 ± 4 Å, again under the condition that the density of the layer was fixed at the same value as that for liquid pentadecane. For such ultrathin layers, it is impossible to fit the thickness and refractive indices simultaneously because the two parameters are interrelated. The adsorption of lysozyme effectively adds a third layer on top of $C_{15}OH$. Assuming that there is no intermixing between lysozyme and the self-assembled monolayer, the extent of protein adsorption can be obtained by adding a third layer into the fitting model. Because of the effect of coupling, the thickness of the protein layer has to be fixed so that the refractive indices can be determined. As lysozyme has a globular dimension of 30 × 30 × 45 $Å^3$, it can be assumed that the layer has the thickness equivalent to one of the axial lengths. If the layer is taken to be 30 Å thick, its refractive index at λ = 4300 Å is fitted to 1.41. If, however, the layer is taken to be 45 Å thick, the corresponding refractive index is 1.38. The surface excess, Γ, can be estimated from the following equation:[16]

$$\Gamma = \frac{d(n_i - n_o)}{a} \quad (1)$$

Here n_i and n_o are the refractive indices for the layer and aqueous solution, d is the lysozyme layer thickness, and a is the refractive index increment and is equal to dn/dc for the protein solution (where c is the lysozyme concentration in bulk solution). For many protein solutions, the value of a is usually around 0.18 cm^3 g^{-1}.[16] It was found that, for both sets of d and n_i, Γ was found to be 1.47 and 1.50 mg m^{-2} and was identical within the experimental error. This result shows that although d and n_i are coupled, Γ is virtually proportional to the product of the two and is therefore not affected.

The variation of Γ for lysozyme adsorption with time at the $C_{15}OH$−solution interface has been studied under different solution pH. The plateau surface excesses after a typical period of 30 min are summarized in Table 1. To

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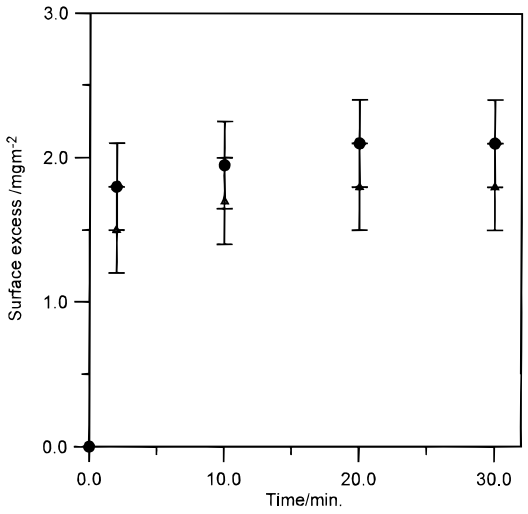

Figure 2. Variation of Γ with time for the adsorption of 4 g dm^{-3} lysozyme at the $C_{15}OH$−water interface under the solution pH of 4 (△) and 7 (●).

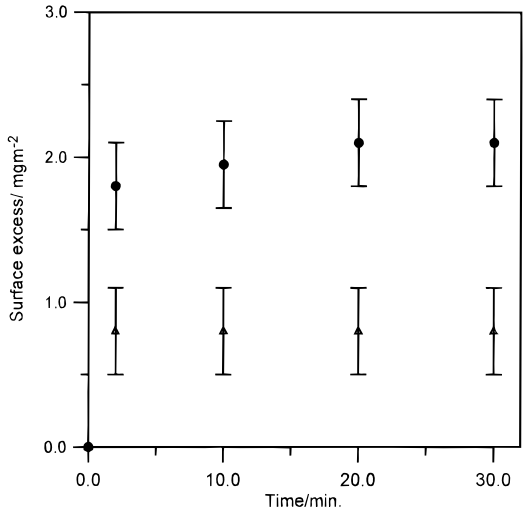

Figure 3. Variation of Γ with time at pH 7 for the adsorption of 0.03 (△) and 4 g dm^{-3} (●) lysozyme at the $C_{15}OH$−water interface.

exemplify the measurement of the effect of pH, we show in Figure 2 the variation of Γ with time at 4 g dm^{-3} lysozyme under pH 4 and 7. It can be seen that for both sets of data the main variation occurs within the first 2 min, followed by a further but minor increment in Γ over next 15 min before constant surface excesses are attained. That the two curves have the same shape suggests that, in spite of the different amount adsorbed, the time-dependent process is not affected by pH. Similar measurements at different fixed lysozyme concentrations were also made. Figure 3 shows the time-dependent adsorption for lysozyme at 0.03 and 4 g dm^{-3} at pH 7. Again, the main change occurs within the first 2 min. After this period, surface excess attains a constant value at the low concentration. However, at the high concentration, a slow but observable increase in Γ is observed after the first 2 min, suggesting that, with the increase in protein concentration, it takes longer for the adsorbed amount to reach a constant value. It should be noted that although the surface excess can attain constant values in minutes, it may take much longer time for the adsorbed molecules to reorient themselves. Hence, fast attainment of constant surface excess does not necessarily warrant the equilibration of the adsorbed layer.

Time-dependent adsorption at the solid−solution interface is a well-known phenomenon, but there is no study available on the kinetic process of lysozyme adsorption at the hydroxyl surface. We note, however, that similar measurements have been done at the bare and methylated silicon oxide−water interfaces using egg white lysozyme and its mutant forms.[17,18] The time scale for reaching saturated adsorption can vary from some 20 min to 2 h, suggesting an average longer period for the adsorbed amount to approach saturation.

(B) Adsorption Measured by Neutron Reflection. Neutron reflection provides better resolution to the structure of the adsorbed protein layer than ellipsometry, partly because neutron beam sources have much shorter wavelengths, which makes neutron measurements more sensitive to the dimension of the adsorbed layer, and partly because the use of isotopic labeling helps to highlight interfacial structure. Although it is difficult to make deuterated proteins, it is rather straightforward to label the $C_{15}OH$ layer and the solvent. In this work we report the measurements at the fully hydrogenated $C_{15}OH$ layer−water interface. The isotopic contrast was achieved by using D_2O.

In a typical neutron reflection experiment, neutron reflectivity, R, is usually measured as a function of κ. R is directly related to the scattering length density profile along the surface normal direction.[19,20] Change in the labeling across the interface can alter R in shape and level, thus highlighting the interface differently. The relationship between the scattering length density of the layer, ρ, and the scattering lengths of the components within the layer can be expressed as

$$\rho = \sum m_i b_i \qquad (2)$$

where m_i is the number density of element i and b_i is its scattering amplitude (scattering length). The scattering lengths are -3.7×10^{-5} Å for H, 6.7×10^{-5} Å for D, 5.8×10^{-5} Å for O, and 6.5×10^{-5} Å for C. This gives a slightly negative scattering length for H_2O and a positive value for D_2O. Change in the ratio of H_2O to D_2O leads to the variation of ρ for the solvent over a wide range, resulting in different reflectivity profiles for the same interface. A neutron reflection experiment was first carried out to determine the thickness and composition of the oxide layer using D_2O and a mixture of D_2O and H_2O with $\rho = 2.1 \times 10^{-6}$ $Å^{-2}$, i.e. contrast matched to Si (CMSi, D_2O:$H_2O \simeq$ 1:2). The reflectivity profiles were analyzed by fitting layer models based on the optical matrix formula. It was found that both reflectivity profiles were best modeled using a thickness τ of 20 ± 3 Å and $\rho = 3.4 \times 10^{-6}$ $Å^{-2}$ for the oxide layer, suggesting a negligible amount of water in the oxide layer. This result is consistent with the measurement from ellipsometry, indicating that the assumption of no defects within the oxide layer is reasonable.

For the hydrogenated $C_{15}OH$ layer, the scattering length is close to zero as a result of opposite signs in scattering lengths for H and C. Simple simulation was done to find out appropriate water contrasts for highlighting the $C_{15}OH$ layer. D_2O was found to be the best solvent, and any addition of H_2O clearly reduced the sensitivity of the measured reflectivity to the structure of the hydrogenated

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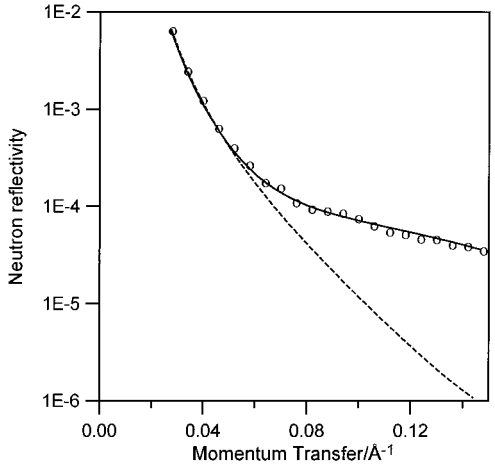

Figure 4. Neutron reflectivity profile measured from the $C_{15}OH-D_2O$ interface. The continuous line was calculated assuming that pentadecyl-1-ol layer is 16 Å thick with liquidlike density. The dashed line is the calculated reflectivity for the bare SiO_2-D_2O interface with the thickness of the oxide layer taken to be 20 Å thick.

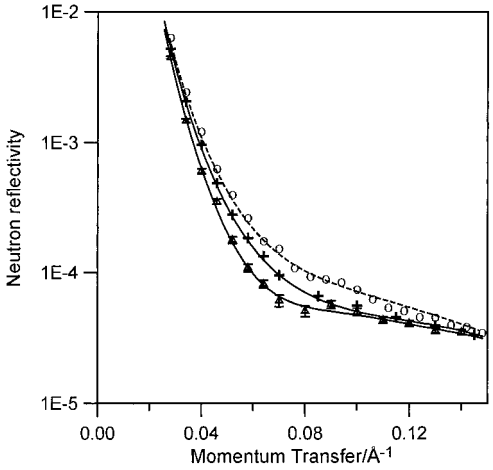

Figure 5. Neutron reflectivity profiles measured from the $C_{15}OH-D_2O$ interface after the adsorption of lysozyme at 0.03 (○), 1 (+), and 4 g dm^{-3} (△) at pH 7. The continuous lines are the best fits with structural parameters listed in Table 2. The dashed line is the best fit to the measured reflectivity before lysozyme adsorption.

layer. This is because the difference in ρ between the layer and the solvent decreases with the increased content of H_2O. Consequently, the structure of the coated $C_{15}OH$ layer was only characterized in D_2O and the resultant reflectivity profile is shown in Figure 4. Also shown in Figure 4 is the reflectivity profile from the bare oxide–D_2O interface and the large difference at $\kappa > 0.08$ $Å^{-1}$ between the two curves indicates the sensitivity of the neutron reflectivity measurement to the organic layer. The best fit (continuous line) was produced with a thickness of 16 ± 2 Å and $\rho = (0.2 \pm 0.1) \times 10^{-6}$ $Å^{-2}$. The errors quoted here reflect the range of variation beyond which obvious deviations between the measured and calculated reflectivities occur. That the thickness of the layer is shorter than the fully extended length of 20 Å for the hydroxyl layer suggests that the layer is in average tilted. The scattering length density for the pure liquid pentadecanol is -2×10^{-7} $Å^{-2}$, and the deviation from this reflects the extent of mixing of water into the layer. The exact composition of the layer can be calculated from ρ for the layer using the following equation:

$$\rho = \rho_a x_a + \rho_w(1 - x_a) \tag{3}$$

Here ρ_a and ρ_w are the scattering length densities for the pure alcohol and D_2O and x_a is the volume fraction of the alcohol chain in the layer. From the fitted value of ρ given above, x_a was calculated to be 0.94 ± 0.05. The result suggests that, within the experimental error, the density of $C_{15}OH$ layer is close to the liquid pentadecanol. It should be noted that, in comparison with the attachment of organic monolayers on gold through thiol reaction (see the work of Prime et al.[21]), the coupling of trichlorosilane groups with the hydroxy groups on the surface of silicon oxide is less selective. The poor binding on silicon oxide has resulted in a less well packed organic layer, evidenced by the high advancing contact angle of 53°, as compared with some 10–30° for the hydroxyl layers coated on gold. Neutron reflection offers a reliable measurement of the average volume fraction of the $C_{15}OH$ layer but is insensitive to the possible presence of defects within the surface region. Other techniques such as IR can offer detailed information about the trans/gauche conformations of the underlying carbon chains on gold but are completely ineffective when such thin layers are grafted on silicon oxide.

Lysozyme adsorption was subsequently carried out in D_2O at pH 7, and the resultant reflectivity profiles are shown in Figure 5. At the lowest concentration of 0.03 g dm^{-3}, a small difference was observed between the reflectivity profiles in the presence and absence of lysozyme, suggesting little lysozyme adsorption. As lysozyme is increased to 1 g dm^{-3}, noticeable deviation occurs around $\kappa = 0.05$ $Å^{-1}$, where a broad interference fringe is clearly visible. Further increase to a lysozyme concentration of 4 g dm^{-3} shifts the broad peak toward lower κ, indicating the thickening of the adsorbed layer and an increased level of adsorption.

Quantitative information about the surface excess of lysozyme was obtained by fitting models to the adsorbed layers. The reflectivity profile at 1 g dm^{-3} was best fitted by a uniform layer distribution with the layer thickness of 38 ± 5 Å. The volume fraction of lysozyme within the layer (x_p) is 0.2 ± 0.04, showing that the adsorbed layer is very loose. Under the condition of uniform layer distribution, Γ can be deduced directly from the layer scattering length density and thickness using

$$\Gamma = \frac{\tau(1 - x_p)}{n_w V_w N_a} \tag{4}$$

where n_w is the number of water molecules associated with each protein molecule, V_w is the water molecular volume, and N_a is Avogadro's constant. The value of n_w can be evaluated from the following equation:

$$n_w = \frac{(1 - x_p)b_p}{\rho V_w - b_w(1 - x_p)} \tag{5}$$

Here b_w and b_p are the scattering lengths for water and protein. Γ was found to be 1.2 ± 0.3 mg m^{-2} for the adsorption at 1 g dm^{-3} under pH 7 and is equivalent to the area per molecule (A) of 2300 ± 200 $Å^2$. Similar treatment has been used to analyze the reflectivity profiles at other concentrations, and the results are given in Table 2. It should be noted that x_p was calculated using eq 3, where x_a is replaced by x_p and ρ_a by ρ_p. For lysozyme in D_2O, ρ_p was taken to be 3.6×10^{-6} $Å^{-2}$. Lysozyme contains

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Table 2. Lysozyme Surface Excesses at the $C_{15}OH$−Water Interface Measured by Neutron Reflection

pH	C/g dm^{-3}	$\Gamma \pm 0.3$/mg m^{-2}	$d \pm 5$/Å
4	0.03	0	
	1	0.5	32
	4	1.2	36
7	0.03	0.4	
	1	1.2	38
	4	2.1	42/30[a]

[a] Denotes a bilayer adsorption.

some 260 labile hydrogens that could exchange with D_2O, and the value of ρ_p used here was calculated assuming complete exchange between labile hydrogens in lysozyme and D_2O. This may not necessarily be so, given that lysozyme is very stable and the exchange of some labile hydrogens may be hindered by hydrophobic encapsulation and the formation of hydrogen bonding within the globular framework. However, we have argued previously that under the experimental conditions the exchange must be fairly complete (over 90%) and the fraction that was not exchanged would be unidentifiable within the experimental error.[4] The uncertainty in the extent of H/D exchange only affects surface excess and does not affect the determination of layer thickness.

In performing the above analysis, we have assumed that lysozyme adsorption did not alter the structure of the oxide and coated organic layers. The difference between the measured profiles with and without lysozyme had to be accounted for by lysozyme adsorption. Lysozyme may mix with the self-assembled monolayer through two possible means: the defects within the $C_{15}OH$ layer or the penetration of the peptide fragments into the pentadecyl chains. The former is unlikely in view of the almost liquid-like density for the self-assembled monolayer. The latter is less obvious on the basis of the results obtained because the isotopic contrasts used are insensitive to the possible mixing between the self-assembled monolayer and peptides as a result of both being hydrogenated. However, in a previous study of lysozyme adsorption onto the hydrophobic OTS−solution interface (OTS was labeled as $Cl_3SiC_{12}D_{24}C_6H_{13}$),[3] no mixing between the OTS layer and peptides was detected, implying that at least there was no penetration of peptide fragments into the inner part of the OTS layer.

We have already indicated that the contact angle of the $C_{15}OH$ surface is about 53 ± 3°, an intermediate value between that for OTS surface (108 ± 3°) and that for hydrophilic silicon oxide (close to 0°). It is worthwhile to compare the physical states of the adsorbed lysozyme on these surfaces. We have previously shown that lysozyme adsorption onto the hydrophobic OTS surface leads to the breakdown of the globular assemblies and the adsorbed layer has to be represented by a fragmented density distribution.[3] On the other hand, at the hydrophilic silicon oxide−water interface, the adsorbed lysozyme molecules retain their globular integrity and the thicknesses of the layers are comparable to the dimension of the globular structure for lysozyme.[1,2] Thus, at pH 7 the thickness of the adsorbed layer is 30 ± 3 Å around a lysozyme concentration of 0.03 g dm^{-3} and is equal to the short axial length, suggesting the formation of sideways-on monolayer. At pH 4, however, the layer is 35 ± 3 Å thick, showing that the adsorbed molecules are on average tilted. As the lysozyme concentration is increased to 1 g dm^{-3}, adsorption leads to the formation of two sideways-on layers at pH 7 and a further tilted monolayer of some 40 Å at pH 4. In comparison, the thickness of 38 ± 4 Å on the $C_{15}OH$ surface at 1 g dm^{-3} tends to indicate the formation

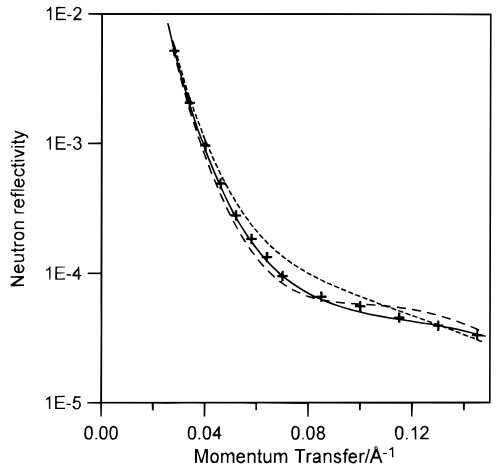

Figure 6. Neutron reflectivity measured from the $C_{15}OH$−D_2O interface after adsorption of 1 g dm^{-3} lysozyme at pH 7. The continuous line is the best fit obtained by taking $d = 38$ Å and $A = 2300$ Å^2. The long dashed line was calculated by assuming that the lysozyme layer is 50 Å thick, and the short dashed line, using the layer thickness of 25 Å.

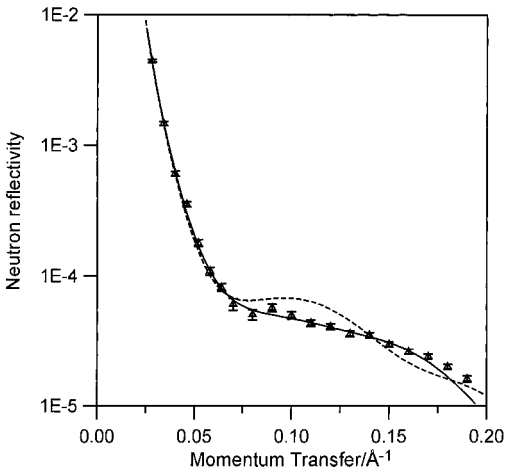

Figure 7. Neutron reflectivity measured from the $C_{15}OH$−D_2O interface after adsorption of 4 g dm^{-3} lysozyme at pH 7. The continuous line is the best two-layer model fit obtained by taking $d_1 = 42$ Å and $A_1 = 1150$ Å^2 and $d_2 = 30$ Å and $A_2 = 6900$ Å^2. The dashed line is the closest uniform layer fit with the thickness of the lysozyme layer taken to be 60 Å.

of a tilted monolayer. That no density gradient is required to fit the data suggests that the adsorbed lysozyme also retains its globular framework. The sensitivity of the measured reflectivity to the thickness of the layer can be tested by varying τ away from the optimal value, while ρ is varied accordingly so that minimum deviation is caused between the calculated and the measured reflectivities. We show in Figure 6 the comparison for the adsorption at 1 g dm^{-3} and pH 7. The long dashed line was calculated by assuming that the layer is 50 Å thick, and the short dashed line, for a layer of 25 Å. The large variation of the shape and level of the reflectivity with layer thickness strongly indicates that the measurement is sensitive to the thickness of the layer within the quoted error of a few angstroms.

The uniform layer model was also used to fit the reflectivity profile at 4 g dm^{-3} and pH 7. The closest fit was calculated using $d = 60$ Å and $\rho = 5.6 \times 10^{-6}$ Å^{-2} and is shown as the dashed line in Figure 7 together with the measured reflectivity profile. It can be seen from Figure 7 that between 0.07 and 0.14 Å^{-1} the calculated reflectivity is higher than the measured one while, above 0.14 Å^{-1},

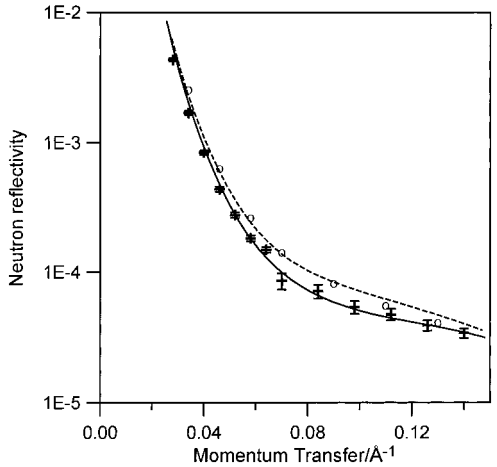

Figure 8. Neutron reflectivity profiles measured from the $C_{15}OH-D_2O$ interface after the adsorption of lysozyme at 1 (○) and 4 g dm^{-3} (+) at pH 4. The continuous line is the best fit to the measured profile at the higher concentration with the structural parameters listed in Table 2. The dashed line is the best fit to the measured reflectivity before lysozyme adsorption.

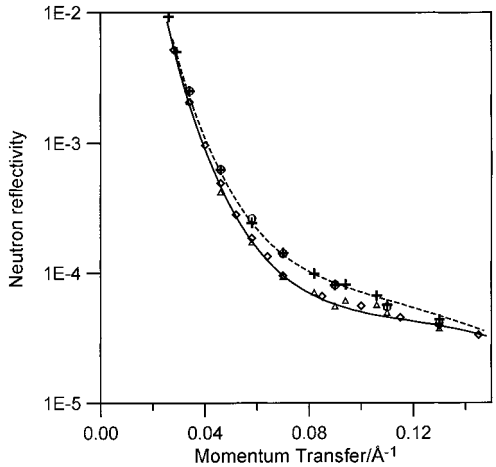

Figure 9. Neutron reflectivity measured at pH 4 (○), followed by increasing pH to 7 (△) and then back to 4 (+), with lysozyme concentration fixed at 1 g dm^{-3}. The reflectivity from the same interface with the initial solution pH 7 (◇) is also shown for comparison. The results show that the reflectivity is only dependent on the final pH and is not affected by the route of pH variation, suggesting that the adsorption is reversible. The continuous line is the best fit to the measurement at pH 7, and the dashed line is the best fit to the reflectivity measured at the interface before lysozyme adsorption. That the dashed line is almost the best fit to the reflectivity profiles measured at pH 4 suggests little adsorption at this pH.

an opposite trend is seen. These deviations clearly indicate that the actual layer density profile does not follow a uniform layer distribution. A two-layer model was subsequently tested, with dense distribution in the inner layer close to the self-assembled monolayer (d_1, ρ_1) and loose distribution in the outer layer close to water (d_2, ρ_2). The best fit was composed of $d_1 = 42 \pm 3$ Å, $\rho_1 = (5.4 \pm 0.2) \times 10^{-6}$ $Å^{-2}$ and $d_2 = 30 \pm 3$ Å, $\rho_2 = (6.1 \pm 0.2) \times 10^{-6}$ $Å^{-2}$, and the calculated profile is shown in Figure 7 as a continuous line. The thickness of the inner layer is just under the long axial length of the globular dimension for lysozyme, suggesting a predominant feature of a headways-on monolayer. The thickness for the outer layer is equivalent to the short axial length, indicating the possible formation of a sideways-on monolayer. An extra piece of information supporting the suggested conformation of the adsorbed layers under these conditions is the area per molecule. A for the inner layer is 1150 ± 50 $Å^2$ and is smaller than the minimum area per molecule of 1350 $Å^2$ (30 Å × 45 Å) required for the formation of sideways-on adsorption but is greater than the limiting value of 900 $Å^2$ (30 Å × 30 Å) required for headways-on adsorption. In comparison, A is 6900 ± 500 $Å^2$ for the outer layer and is significantly greater than the limiting value for sideways-on adsorption.

We have also studied the concentration dependence of lysozyme adsorption at pH 4 under otherwise the same solution conditions, and the resultant reflectivity profiles are shown in Figure 8. Overall, we found that the level of adsorption is substantially reduced as the pH is lowered. No adsorption was detected at 0.03 g dm^{-3}. As the concentration is increased to 1 g dm^{-3}, small difference is observed between the reflectivity profiles in the presence and absence of lysozyme, suggesting weak adsorption. At 4 g dm^{-3}, a visible difference between reflectivity profiles is seen, suggesting an increased adsorption. The thickness of the adsorbed layer at 4 g dm^{-3} was found to be 35 ± 5 Å and is indicative of the formation of a slightly tilted monolayer.

We have shown in our previous work that lysozyme adsorption is irreversible with respect to its bulk concentration at both hydrophilic and hydrophobed solid−water interfaces.[1,2] The same situation has been found at the $C_{15}OH$−water interface, suggesting the relatively high energy barrier for desorption under dilution. The extent of reversibility with respect to pH is however affected by surface hydrophobicity. We found that, for lysozyme adsorbed at the hydrophilic silicon oxide−water interface, the adsorption is reversible with respect to pH over a wide range of concentration. The level of adsorption depends only on the final solution pH, regardless of how it has been reached. At the OTS coated solid−water interface, however, the adsorption is completely irreversible, possibly as a result of the increased contacts among fragmented peptides and between the peptides and the surface. We now extend this work to the surface of $C_{15}OH$ to examine how the intermediate surface hydrophilicitsy affects the extent of reversibility for lysozyme adsorption.

We first examined the reversibility with respect to pH by fixing lysozyme concentration at 1 g dm^{-3}. The measurement was started at pH 4, followed by shifting the pH from 4 to 7 and then back to 4. Figure 9 shows the resultant reflectivity profiles. For comparison, the reflectivity corresponding to the first exposure of the fresh oxide surface to pH 7 is also shown. That the two reflectivity curves at the two pH conditions are identical to each other suggests that the adsorption is completely reversible. A similar experiment has been performed at a higher lysozyme concentration of 4 g dm^{-3}, and the reflectivity profiles are shown in Figure 10. The two reflectivity profiles shown in Figure 10a were obtained at pH 4 and are virtually identical within the experimental error, which would suggest a reversible adsorption. However, there is a large discrepancy between the two reflectivity profiles measured at pH 7 (Figure 10b), suggesting that the adsorption is irreversible. The position of the broad interference fringe corresponding to the shift of pH from 4 to 7 is moved to lower κ, suggesting more adsorption than that measured at initial pH 7. The dashed line shown in Figure 10 represents the reflectivity from the $C_{15}OH$-coated solid−D_2O interface, and the deviation from this curve is indicative of the level of adsorption. It is then clear from Figure 10 that, in spite of the discrepancy between the two reflectivity profiles at pH 7, the overall level of adsorption at pH 7 is greater than at pH 4, a trend

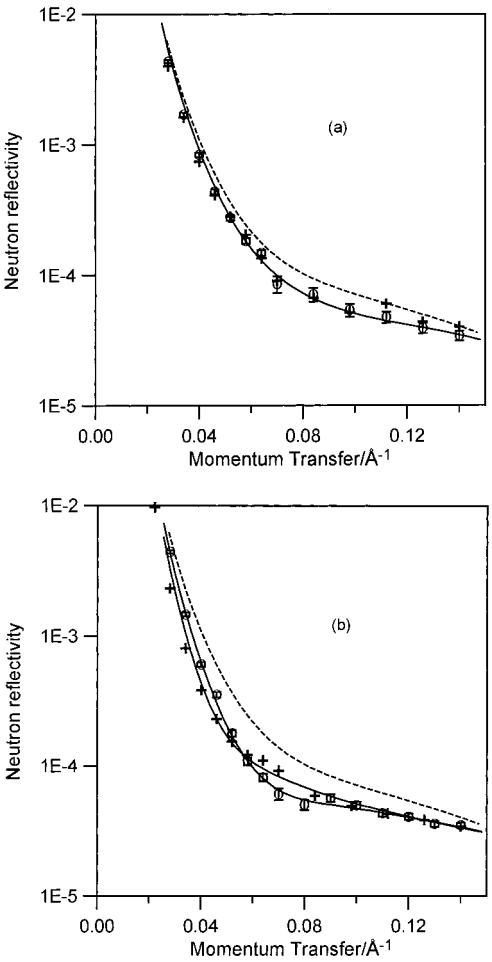

Figure 10. Neutron reflectivity measured at initial pH 4 (○), followed by increasing pH to 7 (+) and then back to 4 (+), with lysozyme concentration fixed at 4 g dm^{-3}. The two reflectivity profiles measured at the initial pH 4 (○) and the value shifted from 7 to 4 (+) are compared in (a). The corresponding two measurements at pH 7 are compared in (b). While the two reflectivity profiles at pH 4 are identical, the two at pH 7 are not, hence indicating irreversible adsorption. The continuous lines are the best fits, and the dashed line is the best fit to the interface before lysozyme adsorption.

similar to that observed at the lower lysozyme concentration of 1 g dm^{-3}.

Discussion

We have summarized lysozyme surface excesses from both ellipsometric measurement and neutron reflection in Table 3. We observed some minor discrepancies in surface excess between the two techniques, and these differences mainly occurred over the low-concentration range. In all cases, however, the differences are under 0.5 mg m^{-2}. Neutron reflection is expected to have better resolution than ellipsometry because the use of D_2O serves to highlight the adsorbed protein layer. The sharp isotopic contrast offers neutron reflection an improved sensitivity to the distribution of the adsorbed protein over low-concentration range. Hence, neutron reflection has been effectively used to validate the ellipsometric results. The overall good agreement between the two techniques suggests that spectroscopic ellipsometry is sufficiently accurate for determining the surface excesses at the modified SiO_2−solution interface, although it cannot offer any information about the dimension of the adsorbed layers.

Table 3. Comparison of Lysozyme Surface Excesses at Different Solid−Water Interfaces[a]

substrate	C/g dm^{-3}	$\Gamma \pm 0.3$/mg m^{-2} (pH 4)	$\Gamma \pm 0.3$/mg m^{-2} (pH 7)
SiO_2	0.03	1.0	1.7
	1	2.1	3.5
	4	3.9	4.7
$C_{15}OH$	0.03	0.2	0.6
	1	0.7	1.3
	4	1.4	2.1
OTS	0.03	1.2	1.9
	1	1.9	3.7
	4	2.6	5.1
PMMA	0.03		0.5
	1		2.0
	4		2.6

[a] The surface excesses on $C_{15}OH$, bare SiO_2, and OTS at 0.03 g dm^{-3} were averaged from neutron and ellipsometry data. The values for PMMA and the rest of OTS surfaces were from ellipsometry measurements only. Neutron data were taken from refs 1−3, and ellipsometry date, from ref 31.

Although many measurements have been made to examine protein adsorption at different interfaces, few results can be directly compared with our data. For example, although lysozyme adsorption on the surface of hydroxyl groups has been studied by Prime et al.[21] using ellipsometry, their measurements have been done at the air−solid interface after the surfaces were rinsed with water and subsequently dried in nitrogen streams. The change in surface excess at the solid−solution interface was inferred from the variation of the thickness of the protein layer left at the air−solid interface. As our results are directly obtained from the solid−water interface, it is not so straightforward to compare the two sets of data. Several ellipsometry measurements have been done at the bare SiO_2−water interface, and the surface excesses were found to be between 2.5 and 3.5 mg m^{-2} for the adsorption of 1 g dm^{-3} lysozyme under the total ionic strength below 0.05 M,[17,22] broadly consistent with the value of 3.5 mg m^{-2} obtained in our previous neutron reflection measurement.[1] There is no literature study of lysozyme adsorption on the surface of OTS, but Malmsten[18] has measured lysozyme adsorption at the methylated SiO_2−water interface using ellipsometry and found a surface excess of 2.3 mg m^{-2} at 1 g dm^{-3} and pH 7.4, close to our value of some 2 mg m^{-2} under similar solution conditions. Some experiments have also been made to quantify the level of lysozyme adsorbed onto various polymer−water interfaces. Lysozyme adsorption onto neutral and charged polystyrene particles has been studied by Haynes et al.[23] and Fair et al.[24] The surface excess was found to be between 2 and 3 mg m^{-2} at a lysozyme concentration around 1 g dm^{-3}. All these results reinforce the fact that the surface excesses on bare oxide and other organic substrates are greater than those adsorbed on the surface of $C_{15}OH$.

We have outlined previously that the level of protein adsorption is determined by the balance of the interactions between the adsorbed protein layer and the substrate and between the protein molecules within the layer.[1−4] The pattern of the variation of surface excess with pH reflects the dominant effect of electrostatic repulsion coupled with steric effects within the adsorbed protein molecules. Since

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the IP for lysozyme is around pH 11, lysozyme is positively charged over the normal pH range. Lowering solution pH away from the IP increases the net charge within the globular framework and hence the lateral electrostatic repulsion. For all the three substrates studied, the surface excesses all show the trend of decreasing with the lowering of pH, supporting the view that surface excess decreases as a result of the intensified lateral electrostatic repulsion.

For the adsorption under given solution conditions, e.g. at a constant pH, the surface excess is always the lowest on the surface of $C_{15}OH$, showing that the hydroxyl surface is most effective at reducing lysozyme adsorption among all the surfaces studied. As the hydroxyl surface has an intermediate contact angle, the results tend to suggest that minimum protein adsorption occurs on the surface with an intermediate hydrophobicity. This statement lends support from the more conventional view about the relationship between surface energy and its efficiency in reducing protein adsorption.[25,26] Much literature work has demonstrated that surfaces with an intermediate energy around 25 mN m^{-1} are most effective at reducing surface fouling. The results shown in Table 3 certainly fit into this empirical rule, but it should be noted that the data on the adsorption at the SiO_2−water interface may be complicated by the presence of surface charges. Iler[27] has shown that, over the normal pH range, the surface of SiO_2 is weakly negatively charged and the charge density is virtually constant between pH 3 and 8. Although we have previously demonstrated that the charge does not affect the main trend of adsorption, it is not clear how this would affect the absolute level of surface excess.

The effect of surface hydrophobicity on protein adsorption has been vigorously examined by Whitesides et al.[21,28] In their work the functionalized surfaces were formed by self-assembly onto Au and Ag through thiol reaction. Lysozyme adsorption on the surfaces with terminal hydroxyl groups, with ethoxylate units bearing terminal OH and OCH_3, and with the mixtures of the ethoxylates and alkyl chains was investigated. Whitesides et al.[21,28,29] found that θ_a for self-assembled monolayers with terminal hydroxyl groups may vary over a wide range, but mostly below 30°, as compared with some 50° observed on silica, suggesting that θ_a is affected by the packing of the coated monolayer and that the layers are overall better packed through thiol reaction than through silane coating. The effectiveness of the hydroxyl surfaces in prohibiting protein adsorption were found to be comparable to those grafted with ethoxylate units. In addition, these authors found that layers coated on the surface of Ag give higher level of trans conformation and hence lower contact angles, indicating better packing on the outer surface by oxygen atoms. The surface excess for lysozyme was, however, found to be much greater on the coated Ag surfaces, suggesting that the surfaces with greater hydrophilicity produce higher surface excesses, a trend similar to the results shown in Table 3. A further piece of information that supports the trend of adsorption is our recent study of protein adsorption on surfaces containing phosphorylcholine (PC) groups.[30−31] For silica surface coated with a thin film of copolymer containing dodecyl chains and PC groups, little lysozyme adsorption was detected. The advancing contact angle on the PC−polymer coated surface was some 70°, again showing a trend of reduced adsorption at the intermediate hydrophobicity. The contact angle of PMMA is about 80 ± 3°, and it can be seen from Table 3 that the surface excesses at the PMMA−solution interface are also lower than the corresponding values on OTS and SiO_2. It should however be noted that the correlation between the level of surface adsorption and surface hydrophobicity through contact angle is very empirical, because contact angle itself offers no information about the structure and chemical nature of the surface. An intermediate value of contact angle can be obtained through different means. For example, a contact angle of 50° can be achieved by coating a loose layer of short chain alkanes, by forming a mixed layer of alkyl chains and hydrophilic groups. It is unclear how the adsorption is affected by the nature of these surfaces under different surface conditions.

The thickness of 38 Å at 1 g dm^{-3} under pH 7 is intermediate between the two axial lengths for lysozyme and is equivalent to the formation of a tilted monolayer. This layer conformation is in contrast to the sideways-on adsorption under the same solution condition at the SiO_2−water interface. There are two main factors contributing to the observed difference. First, the presence of $C_{15}OH$ layer changes the level of van der Waals interaction between the surface and the protein layer.[32] Second, the coating of the $C_{15}OH$ provides a spacing between SiO_2 and the protein layer. As a result, the adsorbed lysozyme molecules are no longer in direct contact with the weakly negatively charged surface and the preferred conformation may result from the reduced electrostatic interaction between lysozyme and the coated surface. As the lysozyme concentration is increased to 4 g dm^{-3}, the density distribution can be modeled into a bilayer. The inner layer is about 42 Å and is thicker than the layer formed at the lower concentration of 1 g dm^{-3}, suggesting that while more lysozyme is adsorbed the layer is further tilted toward a headways-on conformation. The formation of an almost headways-on inner layer is consistent with the area per molecule of 1150 ± 100 $Å^2$, which is smaller than the minimum value required for sideways-on adsorption but above the minimum value required for headways-on adsorption. Shifting pH to 4 causes a substantial reduction in lysozyme adsorption as a result of increased lateral electrostatic repulsion, but the layer thickness is still over 30 Å, implying a similarly tilted conformation. The irreversible adsorption observed at 4 g dm^{-3} when solution pH was shifted from 4 to 7 is likely to be caused by the preadsorbed lysozyme at pH 4. When the pH was increased to 7, the preadsorbed layer was largely retained, thus presenting a surface that was different from the hydroxyl groups. This preadsorbed lysozyme layer must have induced further lysozyme adsorption, resulting in a total amount greater than that obtained at the fresh $C_{15}OH$−solution interface.

Acknowledgment. We thank the U.K. Engineering and Physical Sciences Research Council (EPSRC) for support. E.F.M. thanks Biocompatibles Ltd. for a studentship.

LA991559J

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